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Immunology

Monoclonal antibody-targeted superantigens: A different class of anti-tumor agents

(staphylococcal enterotoxins/cytotoxic T cells/colon cancer cells)

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ABSTRACT The bacterial superantigen staphylococcal enterotoxin (SE) A (SEA) directs cytotoxic T lymphocytes (CTLs) expressing particular sequences of the T-cell receptor (TCR) β chain to lyse tumor cells expressing major histocompatibility complex (MHC) class II molecules, which serve as receptors for SEs. We now report that chemical conjugates of SEA and the colon carcinoma-reactive monoclonal antibodies (mAbs) C215 or C242 mediate T cell-dependent destruction of colon carcinoma cells lacking MHC class II molecules. SEA was covalently linked to the mAbs C215 and C242 via a PEG-based hydrophilic spacer. The C215-SEA conjugate targeted $CD4^+$ as well as $CD8^+$ CTLs to lyse a panel of colon carcinoma cells lacking MHC class II molecules. T-cell recognition of mAb-SEA conjugates was SEA specific, since SEB-selective T-cell lines with potent cytotoxic activity towards Raji cells coated with SEB did not respond to the C215-SEA conjugate. Unconjugated SEA did not induce T-cell lysis of MHC class II^- colon carcinoma cells but efficiently directed CTLs against MHC class II^+ Raji cells and certain interferon-treated MHC class II^+ colon carcinoma cells. These results suggest that SEA-mAb conjugates retain the SEA-related selectivity for certain TCR β-chain variable region (V_β) sequences but, in contrast to unconjugated SEA, mediate the TCR interaction in a MHC class II-independent manner. The cytotoxic activity mediated by C215-SEA and C242-SEA conjugates was blocked by excess of C215 mAb and C242 mAb, respectively, showing that the specificity in the targeting of mAb-SEA conjugates is defined by the antigen reactivity of the mAb. These results demonstrate that bacterial superantigens may be successfully conjugated to mAb with preserved T cell-activating capacity. The circumvention of MHC class II binding of SEs by conjugation to mAb suggests that such conjugates may find general application as antitumor agents, taking advantage of the extreme T cell-activating potency of superantigens.

The collection of superantigens consists of bacterial exoproteins, such as the structurally related staphylococcal and streptococcal exotoxins, but also endogenous self superantigens, including the mammary tumor virus-encoded Mls antigens (1–3). They are characterized by the capacity to stimulate in a major histocompatibility complex (MHC) class II-dependent manner a high frequency of T cells bearing particular T-cell receptor (TCR) β-chain variable segments (V_β) (1, 4–10). Studies on the staphylococcal enterotoxins (SEs) A and B (SEA and SEB) and toxic shock syndrome toxin 1 have shown high-affinity binding to MHC class II molecules (4–6, 10–12). SEA, SEB, and toxic shock syndrome toxin 1 bind to a variety of different MHC class II isotypes and allotypes, and the binding seems to involve conserved peptide sequences expressed on both the MHC class II α and β chains (9–13). T cells recognizing the MHC class II–SE complex are activated to proliferation, cytokine production, and cytotoxicity (14–19). The SE-dependent cell-mediated cytotoxicity (SDCC) results in elimination of MHC class II-expressing SE-presenting target cells (17–19). Studies on fresh MHC class II^+ leukemic cells have suggested that the SDCC mechanisms may be a useful tool for therapeutic elimination of MHC class II^+ tumor cells (16). However, since MHC class II-expressing tumor types only represent a minority of the most frequent human tumors and systemic T-cell activation is expected to result in severe toxicity, it seems reasonable to assume that the SDCC mechanism will not have general application in the treatment of human malignant diseases. During the last decade monoclonal antibodies (mAbs) have been evaluated for tumor therapy, either as native antibodies or conjugated to radioactive isotopes, cytotoxic drugs, or plant toxins (20–23). Recent attempts have also included polyclonal activation of T lymphocytes by antibody heteroconjugates simultaneously recognizing tumor cells and the CD3/TCR complex on T cells (24–26). The direct binding of antibody heteroconjugates to the T cell may be a major drawback *in vivo*, counteracting mAb localization at the tumor site. In this report we demonstrate that conjugates between SEA and mAbs recognizing human colon cancer enable T cells to lyse colon carcinoma cells *in vitro*. The mAb-SEA conjugates direct cytotoxic T lymphocytes (CTLs) against target cells expressing the mAb-defined cell-surface antigen independent of their MHC expression. In contrast to antibody heteroconjugates, the mAb-SEA conjugates do not engage T cells prior to binding to the target cell. We believe that mAb–superantigen-based conjugates represent a novel and powerful approach to tumor therapy, which may have significant advantages in comparison with earlier described antibody-based therapies.

MATERIALS AND METHODS

Reagents. SEB was purchased from Toxin Technology (Madison, WI). Recombinant SEA was expressed in *Escherichia coli* and purified to homogeneity as described elsewhere (ref. 27). The following mAbs were used to detect monomorphic determinants on human MHC class II: HLA-DR, L243 and D1-12; HLA-DP, B7/21; and HLA-DQ, SK10 (HLA-Qw1 and -Qw3), BT3/4 (HLA-DQw1), and SFR16-P1.2 (HLA-DQw2 and -DQw3). The sources of these mAbs have been reported recently (27). The mAbs C215 (IgG2a) and C242 (IgG1) reacting with human colon cancer were obtained from L. Lindholm, Pharmacia Canag (Götcborg, Sweden). Rabbit anti-SEA serum was obtained from Phar-

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Abbreviations: SE, staphylococcal enterotoxin; SEA and SEB, SEs A and B; mAb, monoclonal antibody; TCR, T-cell receptor; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; V_β, β-chain variable regions; SDCC, SE-dependent cell-mediated cytotoxicity; FITC, fluorescein isothiocyanate.

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macia. Fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit and rabbit anti-mouse immunoglobulins were purchased from Dakopatts (Glostrup, Denmark).

Preparation of mAb-SEA Conjugates. Recombinant SEA was coupled to the C215 mAb or C242 mAb by the use of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pharmacia) and a 24-atom-long PEG-based hydrophilic spacer (*N*-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid) as recently described (ref. 28). Briefly, the ε-amino groups of the lysines in the mAb were randomly substituted with the PEG-based spacer, which resulted in 7–18 coupled spacers per mAb. One or two mercapto groups were substituted on the ε-amino groups of lysines in SEA by reaction with the SPDP reagent (29). The spacers ended with reactive iodine groups which reacted with the mercapto groups introduced on SEA, resulting in the formation of stable thioether linkages. The synthesized mAb-SEA conjugate was fractionated on a Superdex 200 HR 16/50 column (Pharmacia) and was eluted with 2 mM phosphate buffer, pH 7.5/0.15 M NaCl. Fractions with the desired product were pooled and analyzed by SDS/PAGE on Phast-Gel5 gradient 4-15 and silver staining (Pharmacia). The conjugates contained zero to three SEA molecules per mAb molecule (average one for C215-SEA and two for C242-SEA).

^{125}I-Labeled SEA (^{125}I-SEA) and C215-^{125}I-SEA Binding Assays. SEA or C215-SEA (5–10 μg) was mixed with 0.5–1.0 mCi (1 Ci = 37 GBq) of ^{125}I (carrier-free NaI, 105 mCi/ml; DuPont/NEN) in 200 μl of phosphate-buffered saline (PBS). One Iodo-Bead (Pierce) was added to the mixture, and after 15 min of incubation at room temperature, 2-mercaptoethanol was added to stop the reaction. Iodinated proteins were separated from free iodine by gel filtration (PD-10 Sephadex G-25M, Pharmacia). When evaluating the inhibition of ^{125}I-SEA and C215-^{125}I-SEA to Raji cells by SEA and C215-SEA, respectively, 10^6 Raji cells in 50 μl of PBS with 1% bovine serum albumin were incubated with various concentrations of unlabeled inhibitor for 30 min at room temperature. Fifty microliters of ^{125}I-SEA and 50 μl of C215-^{125}I-SEA were then added, and the mixture was further incubated for 15 min. Cell-associated radioactivity was separated from free ^{125}I-SEA and C215-^{125}I-SEA by centrifugation of the Raji cells on a 40% Ficoll-Paque (Pharmacia) cushion.

Cell Lines. The B-cell lymphoma line Raji and the colon carcinoma lines SW620, COLO 205, and WiDr were obtained from American Type Culture Collection and cultured in R-medium [RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, and 1 mM pyruvate (Flow Laboratories)]. T-cell lines were established by stimulation of human peripheral blood lymphocytes with SEA or SEB (1 ng/ml) as detailed earlier (15, 16). These T-cell lines were all >99% $CD3^+$. $CD4^+$ and $CD8^+$ sublines were established after separation by positive selection with magnetic beads precoated with anti-CD4 or anti-CD8 mAb (Dynabeads M-450, Dynal A/S, Oslo, Norway). These sublines were >92% pure with respect to CD4 or CD8.

Cytotoxicity Assay. Cytotoxicity was measured at various effector/target cell ratios in a standard 4-hr ^{51}Cr-release assay as described (15). SEs or conjugates were added at various concentrations directly into the assay or were used for preincubation of target cells. Preincubation was performed at 37°C for 30 min followed by extensive washing of the cells.

Analysis by Flow Cytometry. Flow cytometric analyses were performed with indirect immunofluorescence and with standard settings on a FACStarPLUS flow cytometer (Becton Dickinson).

RESULTS

Binding Characteristics of the C215-SEA Conjugate. Binding of mAb C215 and the C215-SEA conjugate to COLO 205 cells, which express the C215 antigen but not MHC class II molecules, was analyzed by flow cytometry with FITC-labeled anti-mouse IgG antibodies. The binding of the conjugate to COLO 205 cells was similar to that of the parental mAb (Fig. 1*A*). Extensive titrations of unconjugated mAb and C215-SEA

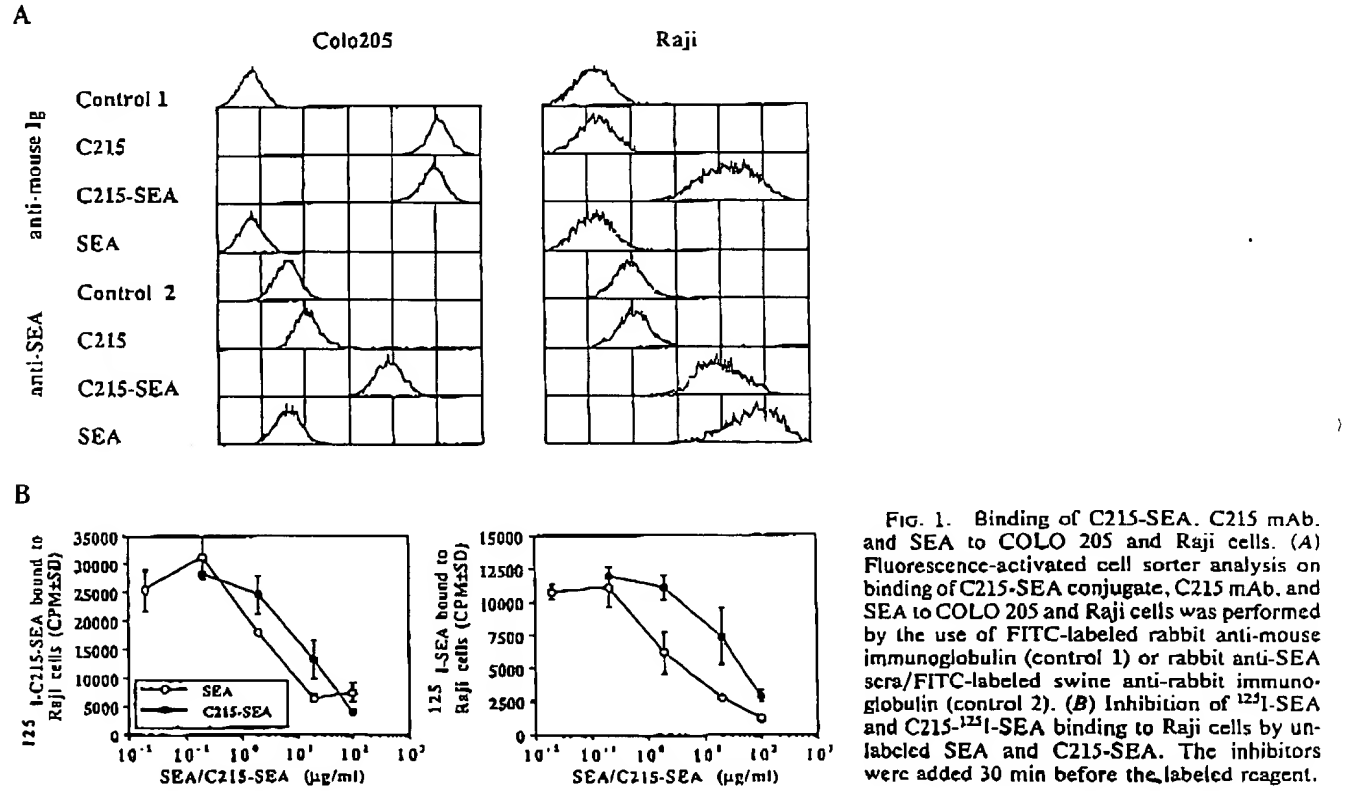

FIG. 1. Binding of C215-SEA. C215 mAb, and SEA to COLO 205 and Raji cells. (*A*) Fluorescence-activated cell sorter analysis on binding of C215-SEA conjugate, C215 mAb, and SEA to COLO 205 and Raji cells was performed by the use of FITC-labeled rabbit anti-mouse immunoglobulin (control 1) or rabbit anti-SEA sera/FITC-labeled swine anti-rabbit immunoglobulin (control 2). (*B*) Inhibition of ^{125}I-SEA and C215-^{125}I-SEA binding to Raji cells by unlabeled SEA and C215-SEA. The inhibitors were added 30 min before the labeled reagent.

conjugate demonstrated similar binding characteristics, with saturated binding detected at about 3 μg/ml (data not shown). The C215-SEA conjugate, but not unconjugated SEA or C215 mAb, was detected on COLO 205 cells as analyzed by the use of rabbit anti-SEA antibodies and FITC-labeled swine anti-rabbit immunoglobulin (Fig. 1*A*). We demonstrated earlier (4) that SEA bound with high affinity to MHC class II molecules on Raji cells. Fluorescence-activated cell sorter analysis showed that SEA and C215-SEA, but not unconjugated C215, bound strongly to Raji cells (Fig. 1*A*). The binding of C215-SEA to Raji cells was efficiently blocked by SEA but not by C215 (data not shown). Thus, in addition to mAb specificity, the C215-SEA conjugate has retained the capacity of SEA to bind to cells expressing MHC class II molecules. To compare the relative MHC class II binding affinity of SEA and C215-SEA, respectively, we utilized a cell-binding assay with ^{125}I-SEA and C215-^{125}I-SEA. Inhibition studies showed that ^{125}I-SEA was displaced in a dose-dependent manner by SEA and C215-SEA mAb (Fig. 1*B*). Similarly, C215-^{125}I-SEA binding to Raji cells was efficiently blocked by SEA and C215-SEA, which indicates that the C215-SEA binding is specific for the MHC class II molecule (Fig. 1*B*). Assuming that the C215-SEA conjugate contains about 15% SEA, the conjugate apparently displays identical MHC class II binding as SEA on a molar basis.

T-Cell Targeting by the C215-SEA Conjugate. The C215-SEA conjugate efficiently directed SEA-responsive CTLs to mediate cytotoxicity against the SW620 colon carcinoma cells, whereas a mixture of unconjugated SEA and C215 mAb had no effect (Fig. 2). Cytotoxicity against SW620 cells was induced by C215-SEA at effector-to-target ratios as low as 3:1, while unconjugated SEA and C215 mAb lacked effect even at 30:1. Lysis was recorded at 3 μg of the conjugate per ml, whereas SEA at 1000-fold higher concentrations only had marginal effects (Fig. 2). The SW620 cell line did not constitutively or after interferon treatment express surface MHC class II molecules, as analyzed by immunoprecipitation and flow cytometry with a panel of mAbs against the HLA-DR, HLA-DP, and HLA-DQ isotypes (27). Furthermore, Northern blot analysis demonstrated absence of HLA-DRα, HLA-DRβ, invariant chain, and HLA-DZα transcripts in SW620 cells (27). C215-SEA conjugate-induced cytotoxicity was observed against several MHC class II$^-$ C215$^+$ colon carcinoma cell lines, including WiDr, COLO 205, and SW620 (Fig. 2). Unconjugated SEA (Fig. 2) and C215 mAb (data not shown) demonstrated only marginal effect on MHC class II$^-$ colon carcinoma cells lines, while SEA induced CTL targeting against MHC class II$^+$ Raji cells (Fig. 2). CTL targeting against Raji cells was efficiently induced by SEA at 0.003 ng/ml, whereas C215-SEA conjugate at 30 ng/ml was required to induce a comparable half-maximal cell lysis, indicating an extremely low efficiency of the C215-SEA conjugate against MHC class II$^+$ C215$^-$ cells compared with SEA.

To analyze the influence of concomitant expression of C215 and MHC class II molecules on a target cell in comparison with separate expression of either of these molecules, we used interferon treatment of COLO 205 cells to induce surface MHC class II expression. Dose–response analysis with untreated MHC class II$^-$ COLO 205 cells showed

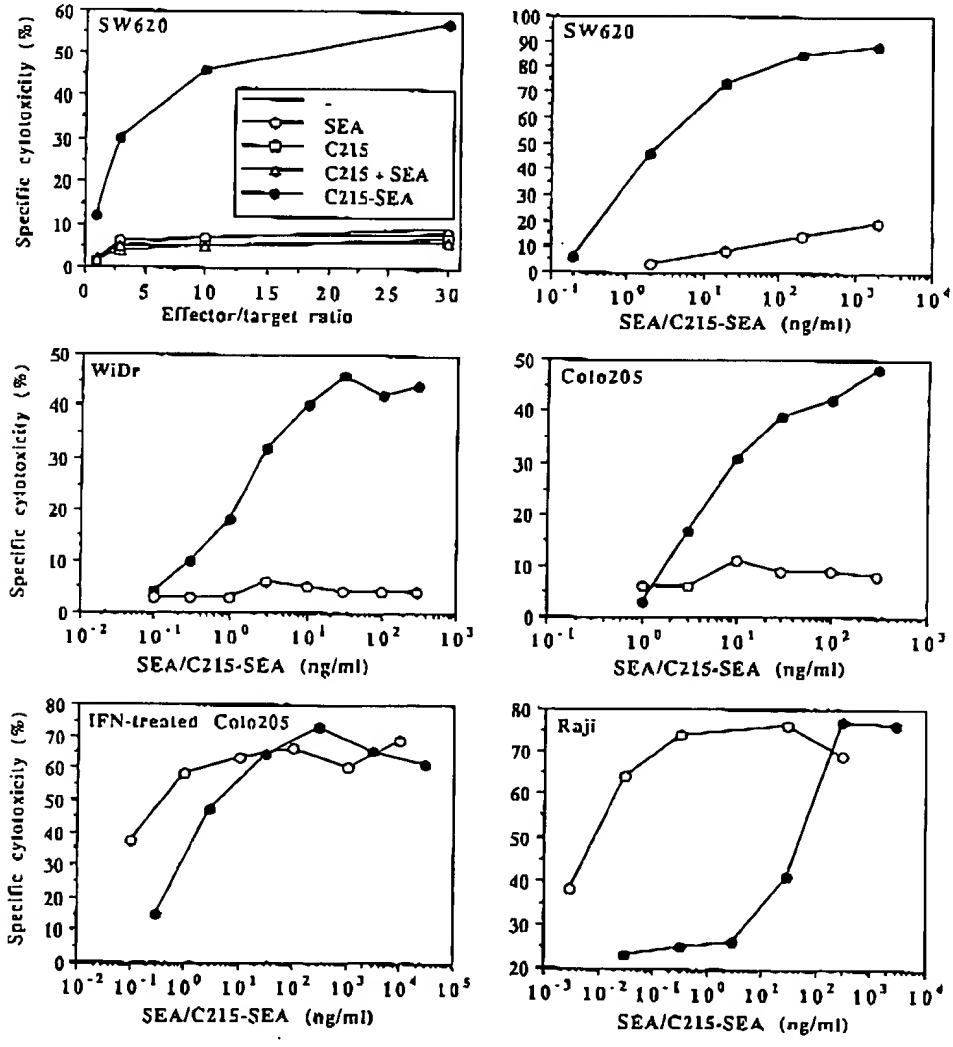

FIG. 2. C215-SEA directs CTLs against MHC class II$^-$ colon carcinoma cells. (*Top Left*) Effect of SEA-responsive CTLs against SW620 cells at various effector-to-target ratios in the absence (−) or presence of C215-SEA, SEA, C215, and a mixture of C215 and SEA (C215+SEA) at a concentration of 1 μg/ml of each additive. Other panels show the capacity of C215-SEA and SEA to target SEA-responsive CTLs against the C215$^+$ MHC class II$^-$ colon carcinoma cell lines SW620, COLO 205, and WiDr; MHC class II$^+$ C215$^+$ interferon-treated COLO 205 cells; and C215$^-$ MHC class II$^+$ Raji cells. Effector-to-target ratio was 30:1. Addition of unconjugated C215 mAb at several concentrations did not induce any CTL targeting against these cell lines. Fluorescence-activated cell sorter analysis on SW620 cells, COLO 205, and WiDr cells using mAbs against HLA-DR, -DP, and -DQ failed to detect any surface MHC class II expression, whereas abundant expression of HLA-DR, -DP, and -DQ was detected on Raji cells and HLA-DR and -DP were detected on interferon-treated COLO 205 cells. COLO 205 cells were treated with 1000 units of recombinant γ interferon per ml for 48 hr prior to use in the CTL assay.

Table 1. $CD4^+$ and $CD8^+$ CTLs lyse colon carcinoma cells presenting the C215-SEA conjugate

Effector*	Target	% cytotoxicity		
		Control	SEA	C215-SEA
$CD4^+$	SW620	2	5	50
$CD4^+$	Raji	0	41	43
$CD8^+$	SW620	0	1	23
$CD8^+$	Raji	2	72	68

*The CTLs (SEA-3) were used at effector-to-target ratio of 30:1 in the absence (control) or presence of SEA and C215-SEA at 1 μg/ml.

sensitivity to lysis at low concentrations of the C215-SEA conjugate but resistance to unconjugated SEA and C215 mAb (Fig. 2). Interferon γ treatment of COLO 205 cells resulted in strong expression of HLA-DR and HLA-DP molecules (27) and sensitivity to CTL lysis at similar concentrations of SEA and C215-SEA (Fig. 2).

C215-SEA Conjugate Targets $CD4^+$ and $CD8^+$ SEA-Responsive CTLs but Not SEB-Responsive CTLs. Both $CD4^+$ and $CD8^+$ CTLs mediated conjugate-dependent cytotoxicity against human colon carcinoma cells (Table 1). Unconjugated SEA failed to induce lysis of SW620 cells but targeted $CD4^+$ and $CD8^+$ CTL against MHC class II^+ Raji cells (Table 1). C215-SEA conjugate efficiently targeted SEA-responsive CTLs against SW620 and Raji cells but failed to target SEB-responsive CTLs (Fig. 3). In contrast, the SEB-responsive CTLs demonstrated strong cytotoxicity against SEB-coated Raji cells (Fig. 3).

Specificity of the mAb-SEA Conjugate Is Defined by the mAb. To demonstrate that the target selectivity of the mAb-SEA conjugate is entirely dependent on the mAb specificity, we performed criss-cross inhibition experiments with unconjugated C215 and C242 mAbs and C215-SEA and C242-SEA conjugates. Cytotoxicity mediated by C215-SEA and C242-SEA was blocked by addition of excess unconjugated C215

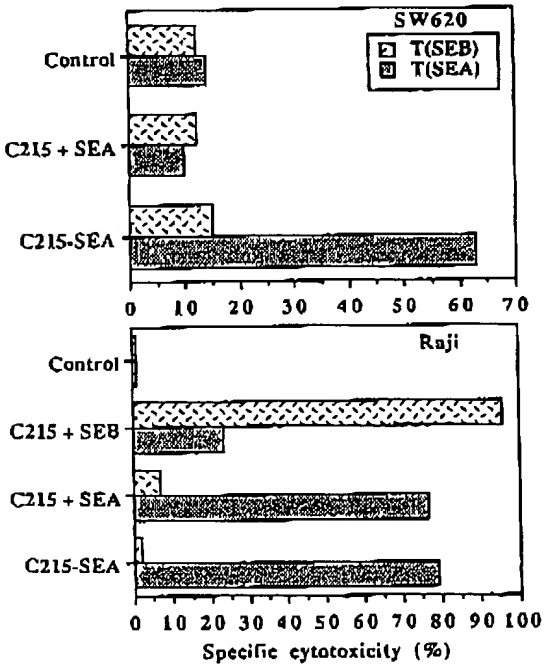

FIG. 3. Lysis of C215-SEA-coated colon carcinoma cells is mediated by SEA but not SEB-responding CTLs. Autologous SEA- and SEB-selective T-cell lines were used at an effector-to-target ratio of 10:1 against SW620 and Raji target cells in the absence (control) or presence of C215-SEA conjugate, a mixture of unconjugated C215 mAb and SEA (C215+SEA), or unconjugated C215 mAb and SEB (C215+SEB) at a concentration of 1 μg/ml of each additive.

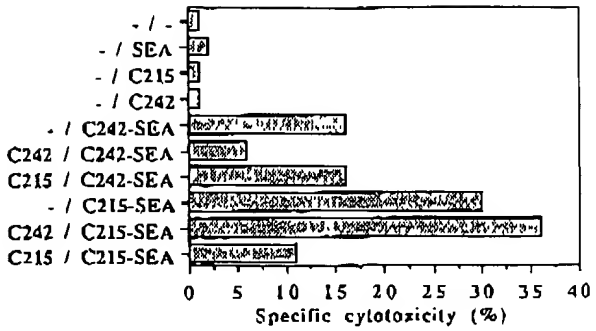

FIG. 4. C215-SEA- and C242-SEA-induced CTL targeting against colon carcinoma cells depends on the antigen selectivity of the mAb. Lysis of COLO 205 cells by a SEA-responsive CTL line in the presence of C215-SEA and C242-SEA conjugates (3 μg/ml) is blocked by addition of unconjugated C215 and C242 mAbs (30 μg/ml), respectively. The unconjugated mAbs or control medium (−) were added to the target cells 10 min prior to addition of the conjugates.

and C242, respectively but was not influenced by addition of the irrelevant mAb binding to the same target cell (Fig. 4) or high concentrations of isotype-specific control antibodies (data not shown).

DISCUSSION

CTLs with specificity for antigens expressed on tumor cells have been demonstrated in patients with malignant melanoma and renal cancer (30, 31). However, they are in most cases infrequent and obviously not capable of protecting the host against the growing tumor. The superantigen SEA allows activation of a frequency of T cells (>10%) even higher than that observed during the response to allogenic MHC in organ transplantation. Since the latter inevitably results in rejection of the transplant, utilization of SEA to direct a high frequency of T cells towards a tumor may hopefully ensure a similar outcome. In this paper we describe such an approach to tumor therapy. Conjugation of SEA to mAb directed against human colon carcinomas provided an agent that was able to selectively target SEA-responsive CTLs against the tumor cells. Earlier studies in our and other laboratories have demonstrated that binding of SEA to MHC class II molecules is a prerequisite for subsequent activation and targeting of T cells (4–6, 15). The SEA-mAb-mediated cytotoxicity apparently is MHC class II independent and does not require antigen-specific effector CTLs. The specificity at the target level is defined by the mAb and at the CTL level by the expression of relevant TCR V_β sequences, suitable for interaction with SEA.

The SW620 colon carcinoma cell line, which lacked mRNA transcripts for HLA-DRα, HLA-DRβ, HLA-DZα, and invariant chain and failed to express surface MHC class II antigens as analyzed by either immunoprecipitation or flow cytometry (27) efficiently presented C215-SEA but not SEA to CTLs. Conjugate-dependent killing not only was restricted to SW620 cells but also was recorded for MHC class II^- COLO 205 and WiDr cell lines as well as freshly isolated MHC class II^- human colon carcinoma cells (data not shown). The existence of MHC class II-independent T-cell activation induced by bacterial superantigens is supported by recent studies by Fleischer and co-workers (32). They demonstrated that SEB bound to silica beads activated T cells, provided that CD8- or CD2-mediated costimulatory signals were delivered. It is reasonable to suggest that soluble SEA interacts with insufficient low affinity to TCR V_β to activate T cells, but when presented in multivalent form on a cell surface or bead, an interaction with enhanced avidity is provided. Delivering costimulatory signals through the adhesion structures

LFA-1/ICAM-1, CD2/LFA-3, or CD8 may play an important role in exceeding the activation threshold (19, 32).

In comparison with unconjugated SEA, the C215-SEA conjugate retains similar MHC class II binding ability but has about 0.1% activity in MHC class II-dependent CTL targeting. This may be interpreted as a possible sterical hindrance by the conjugated C215 mAb when SEA is bound to MHC class II molecules, whereas binding to $C215^+$ on a cell surface by the mAb allows an efficient interaction between SEA and the TCR on the effector cell. In a therapeutic situation, presentation of the conjugate on normal MHC class II-expressing cells such as monocytes, B cells, and activated T cells is undesirable. Binding to normal cells would prevent the conjugate from reaching the tumor, and normal MHC class II^+ cells have been shown to be sensitive to SDCC (16). Although the present conjugation procedure apparently has markedly reduced the SDCC function of the C215-SEA conjugate, it would be of importance to further perturb MHC class II-dependent CTL activity by reducing the binding affinity of the C215-SEA conjugate for MHC class II molecules. We have recently demonstrated that a recombinant C-terminal fragment of SEA contains MHC class II binding determinants (G.H., unpublished data). Similarly, studies on SEC1 and toxic shock syndrome toxin 1 support a C-terminal location for the MHC class II binding epitopes (33, 34). The determination of the amino acids necessary for MHC class II binding may provide a rationale to obtain mAb-SEA conjugates with preserved T cell-activating properties but totally devoid of binding to MHC class II molecules.

Antibody heteroconjugates and hybrid mAbs reacting with tumor cells and epitopes involved in T-cell activation, including the CD3–TCR complex, CD2, or CD28, have been used to target T cells to kill tumor cells *in vitro* (24–26, 35, 36). However, bispecific mAbs have several limitations as therapeutic agents: (*i*) the ability to directly bind to the T cells will ultimately lead to capture of the intravenously administered mAb in peripheral blood and perturb tissue penetration, (*ii*) binding to T cells in the absence of proper cross-linking by the target cell may lead to anergy (37) or cell death (38), and (*iii*) activation of an excessive number of T cells by pan-T-cell heteroconjugates may result in a cytokine-related shock syndrome and suppression of specific immunity dealing with infectious pathogens (39). In contrast, mAb-SEA conjugates do not suffer from these limitations. The ability of the conjugate to efficiently interact with T cells only when bound to the tumor cell surface allows effective tissue penetration and avoids unwanted systemic T-cell activation. Moreover, local production of lymphokines by T cells activated in the tumor area may be expected to result in a beneficial inflammatory response with direct effects on the tumor cells as well as recruitment of new anti-tumor effector cells in a cascade fashion. SEA is an extremely efficient inducer of interleukin 2, tumor necrosis factor, and γ interferon (14, 40). The activation of a limited fraction of T cells bearing the proper TCR V_β sequences preserves a large portion of the T-cell repertoire for dealing with specific immunity. Recently attempts to target antigen-specific $CD4^+$ T cells against tumor cells have been made with conjugates of mAbs and recall antigens such as keyhole limpet hemocyanin (41) and purified protein derivative (42). These mAb–antigen conjugates allowed presentation of relevant processed antigenic peptide fragments on MHC class II molecules and retargeting of CTLs to the tumor cells (41, 42). The recruited T cells are primarily of the CD4 type, and their frequency is significantly lower than those responding to SEA. Moreover, the therapeutic use f such mAb–antigen conjugates is limited to MHC class II^+ tumor cells, and, in contrast to the mAb-SEA conjugates, they do not have a general application for treating MHC class II^- tumors.

The mAb-SEA conjugates described in this study represent a novel class of anti-tumor agents based on conjugation of a superantigen to a tumor-reactive mAb. Development of superantigen–mAb-based agents may serve as an important immunotherapeutic strategy for treatment of malignant diseases that have escaped the host immune response.

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